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Expression of the cadherin-11 gene is a discriminative factor between articular and growth plate chondrocytes[1]

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T. Nakamura M.D., Ph.D.‡ and Dr J. Toguchida M.D., Ph.D.†*
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Summary

Objective: Calcification of hypertrophic chondrocytes is the final step in the differentiation of growth plates, although the precise mechanism is not known. We have established two growth plate-derived chondrocyte cell lines, MMR14 and MMR17, from p53−/− mice (Nakamata T, Aoyama T, Okamoto T, Hosaka T, Nishijo K, Nakayama T, *et al. In vitro* demonstration of cell-to-cell interaction in growth plate cartilage using chondrocytes established from p53−/− mice. J Bone Miner Res 2003;18:97–107). Prolonged *in vitro* culture produced calcified nodules in MMR14, but not in MMR17. Factors responsible for the difference in calcification between the two cell lines may also be involved in the physiological calcification in growth plate.

Design: Gene expression profiles of MMR14 and MMR17 were compared using a cDNA microarray to identify candidate genes involved in the calcification process.

Results: Forty-five genes were identified as upregulated in MMR14, including the cadherin-11 (Cdh-11) gene. The expression of Cdh-11 in MMR14 was detected in cell–cell junctions, while no expression was observed in MMR17. Primary cultured chondrocytes from growth plate (GC) also expressed the Cdh-11, and the staining of Cdh-11 was observed in the late hypertrophic zone of growth plate. Cell aggregation assays showed that chondrocytes required Ca^{2+} to form nodules, and knockdown of the Cdh-11 gene expression using short interfering RNA inhibited the formation of calcified nodules in MMR14. The introduction of Cdh-11 into MMR17 failed to produce calcified nodules indicating that Cdh-11 is one, but not the sole, factor responsible for the production of calcified nodules.

Conclusion: Although the physiological role is still unclear, Cdh-11 is a discriminative factor between articular and growth plate chondrocytes.

Key words: Calcification, Cadherin-11, Growth plate, Chondrocyte.

Introduction

The formation of bone tissues occurs via either a direct (intramembranous) or an indirect (endochondral) process[1], the latter involving exploitation of the unique properties of cartilage and bone. The growth plate cartilage, which is a layered cartilage tissue separating the epiphysis and metaphysis in growing tubular bones, provides a site for physiological endochondral bone formation. Chondrocytes in growth plates undergo proliferation, maturation, hypertrophy, and calcification associated with programmed cell death, and finally are replaced by bone cells. A number of molecules are involved in the development, behavior, and function of growth plate chondrocytes, including, signaling molecules[2–5], growth factors[6–9], and transcription factors[10–12]. It is, however, not yet known how the transit of cells from one zone to the next is regulated. One feasible approach may be to compare gene expression profiles between cells in different zones. Using a combination of laser captured technology and a cDNA microarray, Wang *et al.* compared *in vivo* gene expression profiles between chondrocytes in the proliferating and hypertrophic zone in the growth plate of rat tibiae[13]. The genes for matrix metalloproteinase (Mmp) 13, alpha 1(X) collagen (Col1a1), fibroblast growth factor receptor 1 (Fgfr1), transforming growth factor beta 1-induced transcript and members of the bone morphogenetic protein (Bmp) family were identified as upregulated in hypertrophic chondrocytes[13]. Confirmation of the role of these gene products may require cell lines, derived from each zone, which are extremely difficult to establish from normal animals.

Cells isolated from p53−/− mice are prone to be spontaneously immortalized without losing their differentiated phenotype, and we have established an osteoblast cell line from calvaria[14], and chondrocyte cell lines from articular cartilage[15] and growth plate cartilage[16]. Three cell lines, MMR32, MMR14 and MMR17, were established from costal growth plates of p53−/− mice[16]. Expression profiles of cartilage-related molecules of MMR14 and MMR17 suggested

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that these two cell lines were derived from cells in the hypertrophic zone, and a prolonged culture increased the expression of calcification-related genes in both cell lines[16]. An intriguing difference between these two cell lines is their ability to produce nodules; under standard culture conditions without stimuli specific for calcification, MMR14 produced multiple nodules with signs of calcification, whereas MMR17 formed no nodules and therefore exhibited no signs of calcification[16]. Factors responsible for the difference in calcification between the two cell lines may also be involved in the physiological calcification of hypertrophic chondrocytes. Here we have performed gene expression profiling to identify MMR14- or MMR17-specific upregulated genes. Based on the profiles, the cadherin-11 (Cdh-11: also called OB-cadherin) gene was identified as an MMR14-specific upregulated gene, and further expression and functional analyses suggested that the Cdh-11 gene is one of the factors involved in the process of calcification of growth plate chondrocytes.

Materials and methods

CELL CULTURE AND TISSUE SAMPLES

MMR14 and MMR17 were cultured in DMEM/Ham's F12 (1:1) (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Road Logan, UT) and antibiotics at 37°C in a humidified atmosphere of 5% CO_2/95% air. Calcified nodules were stained with alizarin red as described previously[16]. Tissues were harvested from 4-week-old C57BL/6J mice. Articular cartilage tissues were resected from femora and tibiae, and bone tissues were obtained from calvaria. Growth plate cartilages were dissected from the proximal portion of the cartilage adjacent to the bone tissue under a microscope. Each tissue was properly processed for RNA and protein analyses. The tissues were subsequently cut with scissors into small pieces and dispersed with 0.1% collagenase (Nitta Gelatin, Osaka, Japan). Isolated chondrocytes were cultured in DMEM/Ham's F12 supplemented with 10% FBS. Animal studies were approved by the institutional animal research committee, and performed according to the Guidelines for Animal Experiments of Kyoto University.

GENE EXPRESSION PROFILING

Gene expression profiles of MMR14 and MMR17 were compared using a custom-made cDNA microarray, which consisted of 964 mouse genes of a standard chip (IntelliGene Mouse CHIP Ver. 1, TaKaRa Bio, Shiga, Japan) and an additional 78 bone- and cartilage-related mouse genes as described previously[15]. Both MMR14 and MMR17 cells were cultured for 1 week. Total RNA (20 μg) extracted from cells was labeled by Molony-murine leukemia virus (M-MLV) reverse transcriptase (400 U) (TaKaRa Bio) with fluorescence-conjugated Cy3- or Cy5-dUTP (Amersham Biosciences, Buckinghamshire, UK), respectively. Equal amounts of Cy3- and Cy5-labeled cDNAs were mixed in the reaction buffer (6× SSC with 0.2% SDS, 5× Denhardt's solution, and 0.1 mg/ml denatured salmon sperm DNA), and hybridized to the cDNA chip at 65°C overnight. The chip was washed with 2× SSC with 0.2% SDS twice at 55°C for 5 min and then once at 65°C for 5 min. Finally, the chip was washed with 0.05× SSC at room temperature for 1 min. The hybridized signal was visualized and quantified using the Affymetrix 418 Array Scanner (Affymetrix, Santa Clara, CA) and ImaGene software (BioDiscovery, Marina Del Rey, CA).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Cells were seeded at a density of 3×10^5 cells per 100-mm culture dish, and total RNA was extracted on days 2, 7 and 21 using Trizol Reagent (Invitrogen, Carlsbad, CA), followed by treatment with DNase I (Nippon Gene, Tokyo, Japan). All RT reactions were performed using 1 μg of total RNA with a Super Script First Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer's directions. PCR amplification was carried out using 1 μl of RT product in a final volume of 25 μl containing 20 pmol each of the sense and antisense primers, 25 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U of rTaq polymerase (TOYOBO, Osaka, Japan). All reactions were performed using GeneAmp 9700 (PE Applied Biosystem, Foster, CA). Sequences of primers are listed in Table I. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. The intensity of the amplified fragments of the Cdh-11 and beta-actin genes was evaluated using NIH image and the ratio of Cdh-11 to beta-actin was calculated in each case. PCR experiments for each gene were performed at least three times to confirm the consistency of results. Culture experiments were done at least twice, and the RNA extracted from cells in each experiment was analyzed separately. Tissues from each portion were processed for the isolation of RNA using Trizol Reagent.

WESTERN BLOTTING

Whole cell lysates were prepared from MMR14 cells, MMR17 cells and primary cultured chondrocytes, separated by SDS-PAGE using 10% polyacrylamide gel, and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). The membrane was treated with specific antibodies for Cdh-11 (Zymed Lab, San Francisco, CA) at a dilution of 1:1000 and then with goat anti-rabbit IgG antibody conjugated to horseradish peroxidase as a secondary antibody (Dako, Glostrup, Denmark), and finally visualized using an ECL plus kit (Amersham Biosciences, Buckinghamshire, UK).

IMMUNOHISTOCHEMISTRY

Knee joints were dissected from 4-week-old mice, fixed overnight at 4°C in periodate–lysine–paraformaldehyde solution, and then embedded in paraffin. Longitudinal serial sections were cut at 6 μm, and the immunostaining was performed using the DAKO Envision plus System (Dako). Deparaffinized sections were treated with hydrogen peroxide for 10 min to reduce endogenous peroxidase activity and washed in Tris-buffered saline (TBS). Sections were treated with anti-Cdh-11 antibody at a dilution of 1:100, and further incubated overnight at 4°C. Nonimmune rabbit IgG (Dako) was used as a negative control. Stained slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted in Entellan (MERCK, Darmstadt, Germany).

IMMUNOCYTOCHEMISTRY

MMR14 cells, MMR17 cells and primary cultured growth plate chondrocytes were seeded at a density of 1×10^3 cells per well of a Lab-Tek II chamber slide system (Nalgen Nunc Int. Corp., Rochester, NY). After 7 days of culture, cells were rinsed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde/PBS for 15 min

Table I
Information of primers used in the RT-PCR analyses

Gene	Primers	Size (bp)	Position	Accession no.
Cdh-1 (E-cadherin)	ACGTATCAGGGTCAAGTGCC	376	1268–1287	NM_009864
	CCTGACCCACACCAAAGTCT		1624–1643	
Cdh-2 (N-cadherin)	CAAGAGCTTGTCAGAATCAGG	363	864–884	M31131
	CATTTGGATCATCCGCATC		1209–1227	
Cdh-3 (P-cadherin)	GAGTTTGAGCCGCAGAAGTA	340	1000–1019	X06340
	AACACAGGGGCTTCGTTGAC		1321–1340	
Cdh-4 (R-cadherin)	AAACAGGGGACATCGTGACC	396	1052–1071	D14888
	AAGGCACGGTTCAGCTCATA		1428–1448	
Cdh-5 (VE-cadherin)	TCCGATACGAATACCTGAGC	543	1113–1132	D63942
	TTGCTGTCCTCGTTCTTCAG		1637–1656	
Cdh-6 (K-cadherin)	TTCAAAGACTCGGCCACGGT	335	1090–1119	D82029
	TAGAGGAACGCGGCTGCTTTG		1402–1422	
Cdh-8	ACTCTGGTGGTCTGTCTGGA	601	985–1004	X95600
	AGTTGCCAGTGTTATCTTCC		1567–1586	
Cdh-11 (OB-cadherin)	TCTGTATCAGAAGCAGCTGTC	729	1095–1115	D21253
	TCCTGGTCATCTGCACTAAC		1806–1825	
Cdh-15 (M-cadherin)	CCCTGGACTATGAGAGCCGT	645	1498–1517	M74541
	GCATGGCTCACGTTAATCTG		2124–2143	
Col2a1	CACACTGGTAAGTGGGGCAAGACCG	173	31276–31300	M65161
	GGATTGTGTTGTTTCAGGGTTCGGG		31448–31424	
Col10a1	ATGCCGCTTGTCAGTGCTA	625	6670–6688	X67348
	GATCCTCACATACCCACTG		7294–7276	
Aggrecan	GTCCCTGGTCAGCCCCACTTG	636	1561–1581	NM_007424
	CACTGACACACCTCGGAAGCA		2176–2196	
Pthrpr	TGCTTGCCACTAAGCTTCG	214	1207–1225	X78936
	TCCTAATCTCTGCCTGCACC		1451–1470	
Alkaline phosphatase	GCCCTCTCCAAGACATATA	363	478–486	J02980
	CCATGATCACGTCGATATCC		821–840	
Osteoblastic specific factor 2 (Osf2)	ATGCTTCATTCGCCTCACAAAC	655	1–22	AF010284
	CTACAACCTTGAAGGCCACG		636–655	
Osteocalcin	TCTGACAAAGCCTTCATGTC	199	136–155	X04142
	AAATAGTGATACCGTAGATGCG		313–334	
Bsp1/Opn	ACACTTTCACTCCAATCGTCC	240	608–628	X16151
	TGCCCTTTCCGTTGTTGTCC		828–847	
Bsp2	TCGAAGAATCAAAGCAGAGGA	156	151–171	NM_008318
	CTATCGCAGTCTCCATTTTC		287–306	
Osterix	TTGAGGAAGAAGCTCACTATGGC	390	119–139	AF184902
	TGTACCACGAGCCATAGGGATG		487–508	
β actin	AAGAGAGGTATCCTGACCCT	218	261–280	X03672
	TACATGGCTGGGGTGTTGAA		458–478	

All primer sequences are written from 5′ to 3′. For each primer pair, the top sequence is sense and the bottom is antisense.

at room temperature, and rinsed three times with TBS. The samples were treated with hydrogen peroxide for 10 min to reduce endogenous peroxidase activity and washed in TBS. Samples were treated with anti-Cdh-11 antibody at a dilution of 1:500, and further incubated overnight at 4°C. Nonimmune rabbit IgG was used as a negative control. After three washes in TBS, samples were treated with a 1:100 dilution of fluorescent-conjugated anti-rabbit antibody at 37°C for an hour. Stained slides were washed with TBS, counterstained with DAPI, mounted with cover slips, and observed with a confocal laser scanning microscope.

CELL AGGREGATION ASSAY

The cell aggregation assay was performed using the original method[17]. After 2 weeks in culture, monolayer cells were dissociated with 0.01% trypsin in the presence (TC treatment) or absence (TE treatment) of 1 mM $CaCl_2$ for 30 min at 37°C. After being washed with HEPES-buffered Ca^{2+}, Mg^{2+}-free Hanks' solution (HCMF), the cells were allowed to aggregate in HCMF with or without 1 mM $CaCl_2$ and shaken for 60 min at 37°C.

CONSTRUCTION OF RETROVIRAL VECTOR AND ESTABLISHMENT OF STABLE CELL LINE

Full-length murine Cdh-11 cDNA kindly provided by Dr Takeichi was subcloned into the pQCXIH retrovirus vector (Invitrogen), and the recombinant virus (pQCXIH-Cdh11) was transfected into Ampho293 cells by lipofection using Lipofectoamine Plus (Invitrogen), and the culture supernatant was harvested at 48 h posttransfection. One milliliter of fluid was added to MMR17 cells in the presence of polybrene (6 mg/ml), and a stable cell line was obtained after 3 weeks of culture under drug selection.

CONSTRUCTION AND TRANSDUCTION OF LENTIVIRAL VECTORS EXPRESSING SHORT INTERFERING RNA

The CMV promoter of pLenti6/V5-DEST (Invitrogen) was removed by digestion of ClaI and AgeI, and the cut end was blunted, into which the Gateway recipient cassette rFA (Invitrogen) was inserted. Human U6 promoter was transferred into SalI and NotI sites of pENTR1A (Invitrogen), and the short hairpin oligonucleotides were

Table II
Genes upregulated in MMR14 and MMR17

	Upregulated genes in MMR14	Gene symbol	Accession no.	Upregulated genes in MMR17	Gene symbol	Accession no.
Extracellular cell matrix	Procollagen, type I, alpha 1	Col1a1	BC050014	Osteopontin	Opn	AF515708
	Procollagen, type XI, alpha 1	Col11a1	NM_007729	Matrix gamma-carboxyglutamate (gla) protein	Mglap	NM_008597
	Fibronectin 1	Fn1	NM_010233	Annexin A5	Anxa5	NM_009673
	Fibulin-1	Fbln1	NM_010180	Syndecan 2	Sdc2	NM_008304
	Tenascin C	Tnc	NM_011607	Selenoprotein P, plasma, 1	Sepp1	NM_009155
	Laminin, gamma 1	Lamc1	NM_010683			
	Thrombomodulin	Thbd	NM_009378			
ECM-related enzyme	Alkaline phosphatase 2	Alp2	NM_007431	Ectonucleotide pyrophosphatase/ phosphodiesterase 2	Enpp2	NM_015744
	Matrix metalloproteinase 13	Mmp13	NM_008607			
	Calpain 6	Capn6	NM_007603			
Cell surface marker	CD59	Cd59	U60473	Milk fat globule-EGF factor 8 protein	Mfge8	NM_008594
	Neogenin	Neo1	NM_008684	CD97	Cd97	NM_011925
	Cadherin-11	Cdh-11	NM_009866	CD151	Cd151	NM_009842
	Immunoglobulin superfamily containing leucine-rich repeat	Islr	NM_012043	Integrin beta 1	Itgb1	NM_010578
				Colony stimulating factor 1 (macrophage)	Csf1	NM_007778
				Small inducible cytokine A9	ScyA9	AF128195
Cytoskeleton-related	Myosin IB	Myo1b	NM_010863	Cofilin 2	Cfl2	BC007138
	Neural precursor cell expressed developmentally downregulated gene 5	Nedd5	D49382	Neurofibromatosis 2	Nf2	NM_010898
	KAP3B	Kap3	D50367			
Signal molecule	Parathyroid hormone/parathyroid hormone related peptide receptor	Pthrpr	X78936	Transforming growth factor, beta receptor II	Tgfbr2	BC016262
	Fibroblast growth factor receptor 1	Fgfr1	NM_010206	Calcium/calmodulin-dependent protein kinase II, delta	Camk2d	NM_023813
	Transforming growth factor beta 1 induced transcript 1	Tgfb1i1	NM_009365	Harvey rat sarcoma virus oncogene 1	Hras1	NM_008284
	MAD homolog 3	Smad5	NM_016769	Dual specificity phosphatase 1	Dusp1	NM_013642
	Bone morphogenetic protein 4	Bmp4	NM_007554	RhoC	RhoC	AK011599
			U28423	G protein, gamma 2 subunit	Gng2	NM_010315
				Neuroblastoma, suppression of tumorigenicity 1	Nbl1	NM_008675
				MARCKS-like protein	Mlp	NM_010807
				Heat-responsive protein 12	Hrsp12	NM_008287
Cytoplasmic protein	Phosphoprotein enriched in astrocytes 15	Pea15	NM_011063	N-myc downstream regulated gene 1	Ndrg1	NM_010884
	Protein kinase inhibitor p58	Prkrir	NM_028410	Homer homolog 3	Homer3	NM_011984
Metabolic enzyme	Ornithine decarboxylase, structural 1	Odc1	NM_013614	Glutamate oxaloacetate transaminase 2	Got2	NM_010325
	Methylenetetrahydrofolate dehydrogenase (NAD+ dependent)	Mthfd2	NM_008638	Aldolase 1, A isoform	Aldoa	NM_007438
				Glucosidase, alpha, acid	Gaa	NM_008064
				Lithium-sensitive myo-inositol monophosphatase A1	IMPA1	AF042730
Transcription factor	SRY-box containing gene 9	Sox9	NM_011448	Peroxisome proliferator activated receptor gamma	Pparg	NM_011146
				High mobility group AT-hook 1	Hmga1	NM_016660
				c-jun protein oncogene	c-jun	J04115

Cell cycle regulator	Cyclin D	Cyc1	M64403	Cp	NM_007752
	Cyclin-dependent kinase 2	Cdk2	NM_016756	Brf1	NM_028193
	Cell division cycle 2 homolog A	Cdc2a	NM_007659	Ddx5	NM_007840
	Proliferating cell nuclear antigen	Pcna	X57800	Ndufa1	NM_019443
Secretory protein	Reticulocalbin	Rcn1	D13003	Ceruloplasmin		
RNA-related molecule	Growth arrest specific 5	Gas5	NM_013525	Butyrate response factor 1		
				DEAD (Asp-Glu-Ala-Asp) box polypeptide 5		
Mitochondrial protein				NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1		
				ATPase, Na+/K+ transporting, beta 3 polypeptide	Atp1b3	NM_007502
				Branched chain ketoacid dehydrogenase E1, alpha polypeptide	Bckdha	NM_007533
				Acetyl-Coenzyme A dehydrogenase, medium chain	Acadm	NM_007382
				ATPase, H+ transporting, V1 subunit B, isoform 2	Atp6v1b2	NM_007509
				ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	Atp5f1	NM_009725
	12 ESTs were also upregulated			Six ESTs were also upregulated	Six ESTs were also upregulated	

inserted at the 3′ site of the U6 promoter. The sequences of the short hairpin oligonucleotides for Cdh-11 are sense 5′-GCCCCAAGTTATATCCATGAGGAAGCTTGGTCATGGATATAACTTGGGGCTTTTTGC-3′ and the antisense 5′-GGCCGCAAAAAGCCCCAAGTTATATCCATGACCAAGCTTCCTCATGGATATAACTTGGGGC-3′. As a negative control, short hairpin oligonucleotides for the firefly luciferase gene were used. The sense sequence was 5′-TCGATGTACACGTTCGTCACGAAGCTTAGTGACGAACGTGTACATCGACTTTTTGC, and the antisense sequence was 5′-GGCCGCAAAAAGTCGATGTACACGTTCGTCACTAAGCTTCGTGACGAACGTGTACATCGA. Then the Gateway Technology recombined the entry vector with the lentiviral vector. The lentiviral siRNA vector for Cdh-11 was designated LV-Cdh11i and the lentiviral siRNA vector for luciferase was designated LV-FFi. Lentiviral supernatants were produced according to the manufacturer's directions (Invitrogen). For transduction, 1×10^5 MMR14 cells were seeded per well of a 6-well plate and infected after 24 h. MMR14 cells were incubated with 1 ml of unconcentrated virus containing 4 μg/ml of polybrene. Transduced cells were cultured in DMEM/Ham's F12 with 10% FBS for 21 days. For cloning, the transduced cells were treated with 2 μg/ml of blasticidin. After 2 weeks of culture, antibiotic-resistant colonies were removed and expanded for assay. Calcified nodules were stained with alizarin red. The number of calcified nodules was manually enumerated under a microscope, and the calcified area was analyzed with NIH image.

STATISTICS

Results are expressed as the mean ± S.E. Statistical analysis was performed with Student's *t* test. A significant difference was accepted at the $P < 0.05$ level. Each experimental group was compared with its own control, which was prepared and analyzed simultaneously.

Results

IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED BETWEEN MMR14 AND MMR17

Genes differentially expressed between MMR14 and MMR17 were identified using the cDNA microarray. Forty-five genes were identified as upregulated in MMR14, their expression being more than twofold that in MMR17 (Table II). Among 45 upregulated genes, 12 were expressed sequence tags (ESTs), and 33 were known genes, some of which have been shown to promote calcification or aggregation in growth plates, such as the SRY-box containing gene 9 (Sox9), Col1a1, alkaline phosphatase (Alp), Bmp4 and Mmp13 genes. Using equivalent criteria, 48 genes were identified as in MMR17 cells, their expression being more than twofold that in MMR14 (Table II). Among them, six were ESTs and 42 were known genes, some of which have been shown to prevent calcification or aggregation, such as the matrix gamma-carboxyglutamate (gla) protein (Mglap), CD151 and ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2) genes.

Among MMR14-specific upregulated genes is the gene for Cdh-11, which has been recognized as an osteoblast-specific cadherin[18], and shown to regulate the cell–cell adhesion and nodular formation of osteoblasts, which is important for mineralization of the osteoid matrix[19,20]. The role of cartilage tissues, however, was never

analyzed, and therefore we focused on the Cdh-11 gene in this study.

CONFIRMATION OF THE RESULTS OBTAINED WITH THE cDNA MICOARRAY

To confirm the results obtained with the cDNA microarray, mRNA expression of the Cdh-11 gene in MMR14 and MMR17 was analyzed by semiquantitative RT-PCR. The expression of the Cdh-11 gene increased in a time-dependent manner in MMR14 cells, but was not detected in MMR17 cells at any of the time points [Fig. 1(A)]. The level of Cdh-11 protein expression was well correlated with the level of mRNA expression [Fig. 1(B)], and the immunocytochemical analysis clearly showed that Cdh-11 was expressed in areas of cell–cell junctions in MMR14 after 1 week of culture, but was not observed in MMR17 [Fig. 1(D)]. These results confirmed the result of the gene expression profiling.

Cdh-11 IS EXPRESSED IN NORMAL CHONDROCYTES DERIVED FROM GROWTH PLATE CARTILAGE

To investigate whether Cdh-11 is expressed in normal chondrocytes in growth plates, primary cultured chondrocytes from costal growth plates (GC) were analyzed as well as primary cultured chondrocytes from articular cartilage (AC) and primary cultured osteoblasts from calvaria (CO). The expression of the Cdh-11 gene was also detected in CO as well as in GC, but not in AC [Fig. 2(A), center panel]. Expression of the Col10a1 and parathyroid hormone related peptide receptor (Pthrpr) genes, which were considered as markers of growth plates, was detected only in GC and not in AC or CO [Fig. 2(A), left panel]. On the other hand, the expression of osteocalcin, osteopontin (Opn), and bone sialoprotein 2 (Bsp2) genes, which were considered as markers of osteoblasts, was not detected in GC [Fig. 2(A), right panel], suggesting that the contamination of osteoblasts in GC was minimal. The level of Cdh-11 protein expression was upregulated during culture

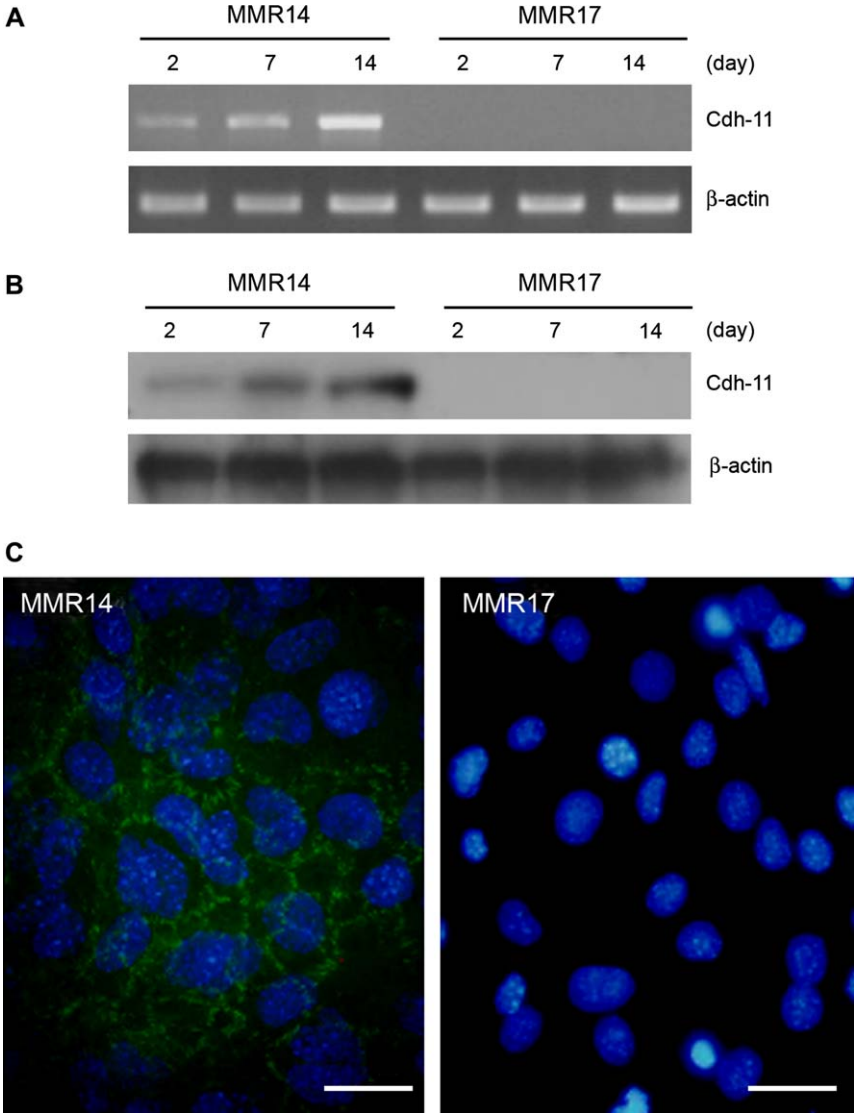

Fig. 1. Expression of Cdh-11 in MMR14 and MMR17. The expression of Cdh-11 in MMR14 and MMR17 cells during culture was examined by RT-PCR (A) and Western blotting (B). Localization of Cdh-11 was analyzed by immunocytochemistry (C). Counterstained with DAPI (original magnification, $\times 400$; bar $= 10\ \mu M$).

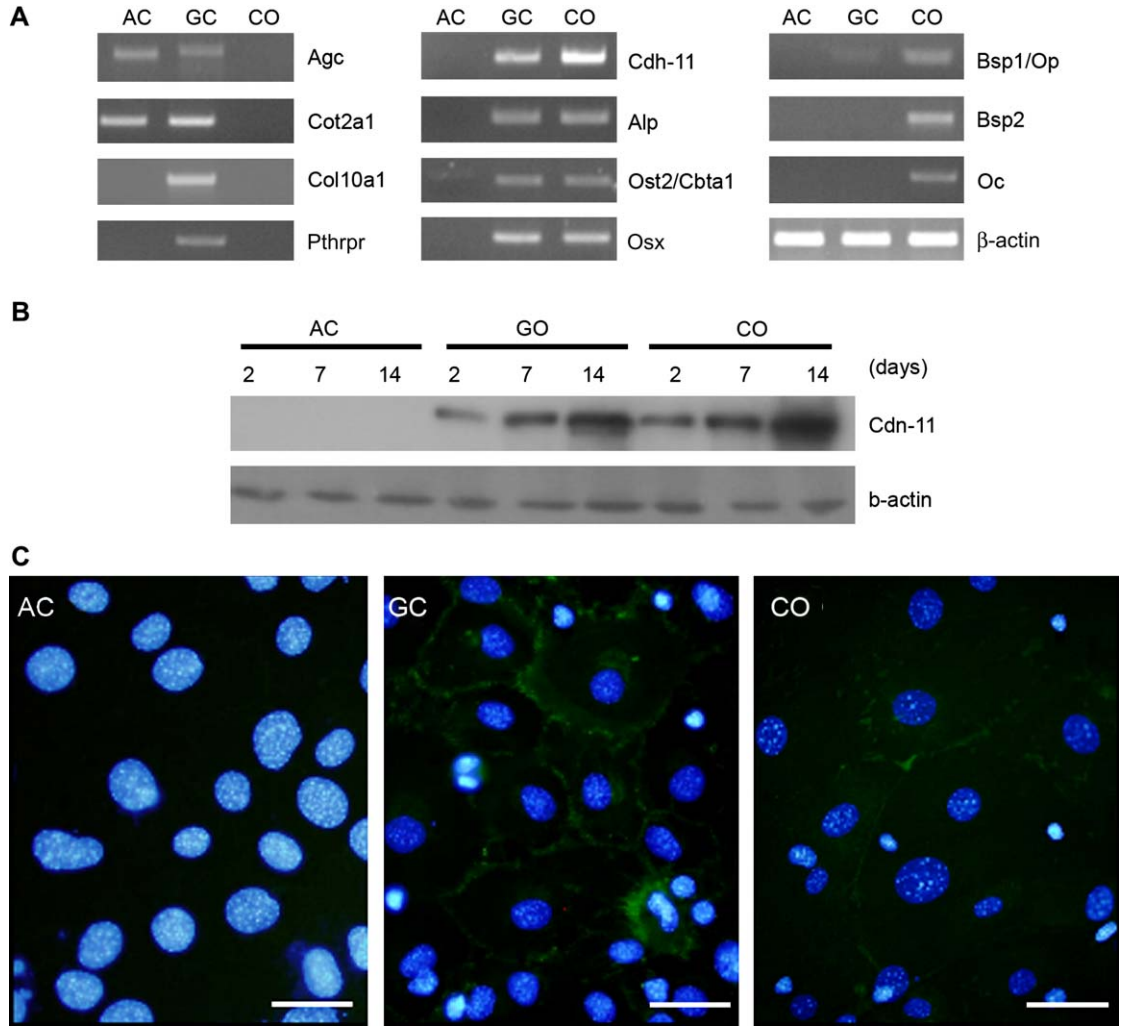

Fig. 2. Expression of Cdh-11 in primary cultured articular chondrocytes, growth plate chondrocytes and COs. The mRNA expression of Cdh-11 and bone/cartilage-related genes in primary cultured articular chondrocytes, growth plate chondrocytes and COs was examined by RT-PCR (A). The expression of Cdh-11 protein in these primary cultured cells was analyzed by Western blotting (B) and immunocytochemistry (C). Counterstained with DAPI (original magnification, $\times$400; bar = 10 μM).

in GC as well as in CO [Fig. 2(B)], and immunocytochemical analysis showed that Cdh-11 protein was properly expressed in areas of cell–cell junctions in GC and CO, but not in AC [Fig. 2(C)].

Cdh-11 IS EXPRESSED *IN VIVO* IN GROWTH PLATE CARTILAGE

Localization of Cdh-11-positive cells in growth plate was investigated by the immunohistochemical analysis. Positive staining for Cdh-11 protein was observed in cells from the late hypertrophic zone to calcified zone of mouse growth plate cartilage (Fig. 3).

EXPRESSION OF THE CADHERIN GENE FAMILY IN CHONDROCYTES

Cadherin genes compose a large family, and some may be functionally redundant[21,22]. The expression of some classical cadherin genes was analyzed by RT-PCR in cell lines and primary cultured cells (Fig. 4). The cadherin-2 (Cdh-2, N-cadherin) and cadherin-3 (Cdh-3, P-cadherin) genes were expressed in cell lines (MMR14 and MMR17) (Fig. 4, left panel), and also in primary cultured cells (AC, GC, and CO) (Fig. 4, right panel). The cadherin-5 (Cdh-5, VE-cadherin) was negative in cell lines, whereas it was detected in primary cultured GC and CO, presumably due to contaminated endothelial cells in primary cultured cells. Therefore, among the classical cadherin genes analyzed, only the Cdh-11 gene showed a difference between MMR14 and MMR17, which also showed a difference between AC and GC.

CELL–CELL ADHESION OF MMR14 AND PRIMARY CULTURED CHONDROCYTES REQUIRES CA^{2+}

To examine the dependence of MMR14 cells on Ca^{2+} for cell–cell adhesion, a cell aggregation assay was performed. At day 14 of culture, MMR14 cells produced a number of nodules. When trypsinization was carried out with $CaCl_2$ (TC treatment), cells in nodules were resistant to dissociation ([Fig. 5(A, *a*)], whereas the trypsinization without

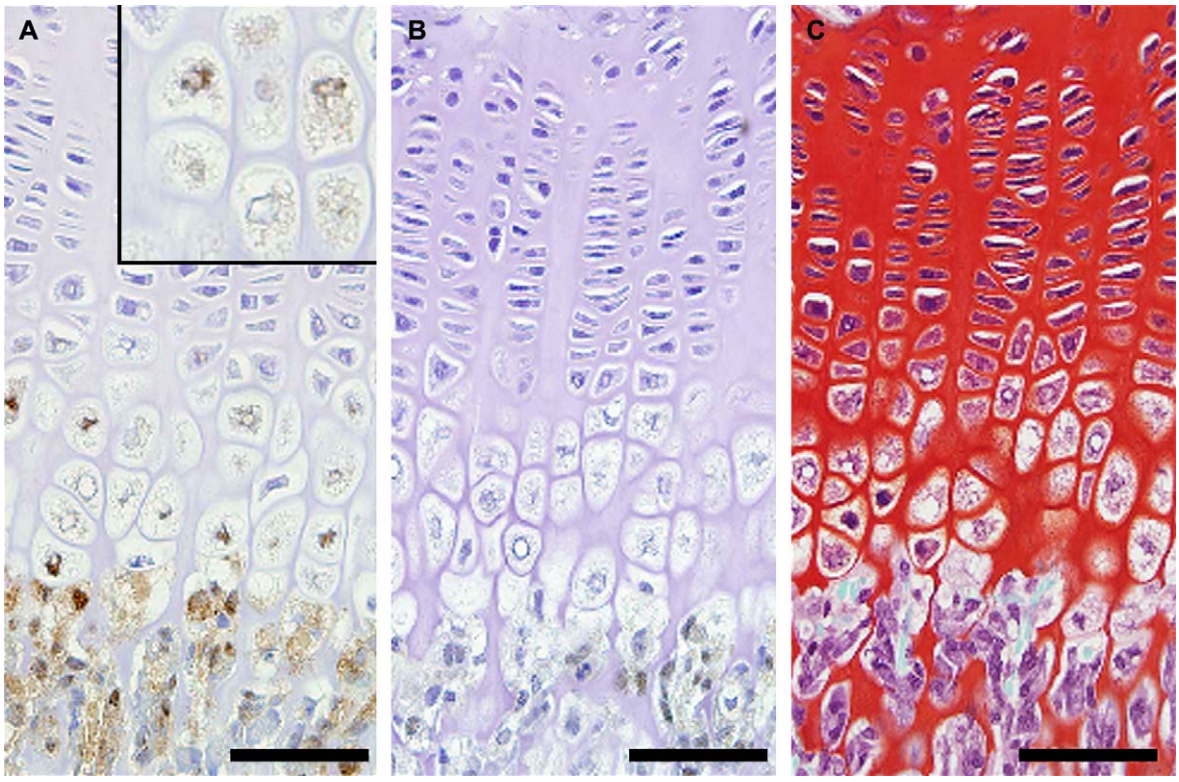

Fig. 3. Expression of Cdh-11 in growth plate. Localization of Cdh-11 in growth plate was analyzed by immunohistochemistry. Decalcified specimens were stained with antibody for Cdh-11 (A) or nonimmune IgG (B). Same specimens were also stained with Safranin-O (C). Each specimen was counterstained with hematoxylin (original magnification, ×400; bar = 50 μM).

$CaCl_2$ (TE treatment) dispersed the nodules [Fig. 5(A, *b*)]. The number of alizarin red-positive nodules after TC treatment [Fig. 5(B, *a*)] was much higher than that after TE treatment [Fig. 5(B, *b*)], and the difference was statistically significant [Fig. 5(C)]. These results suggested that MMR14 cells required Ca^{2+} to maintain the nodular aggregation.

After being dispersed by trypsinization without $CaCl_2$ (TE treatment), cells were allowed to reaggregate in HCMF with [Fig. 5(D, *a*, *c*, and *e*)] or without [Fig. 4(D, *b*, *d*, and *f*)] $CaCl_2$. MMR17 cells showed no aggregation irrespective of the presence of $CaCl_2$ [Fig. 5(D, *a* and *b*)]. On the other hand, MMR14 cells attached to each other in the presence [Fig. 5(D, *c*)] but not absence [Fig. 5(D, *d*)] of $CaCl_2$. Similar results were obtained with primary cultured GCs, which showed aggregation when incubated with [Fig. 5(D, *e*)] but not without [Fig. 5(D, *f*)] $CaCl_2$. These results indicated that the cell−cell adhesion of MMR14 and primary cultured chondrocytes depends on factors requiring Ca^{2+}, suggesting the involvement of cadherins, and the significant difference between MMR14 and MMR17 cells strongly suggested that Cdh-11 is the cadherin gene responsible for this phenomenon.

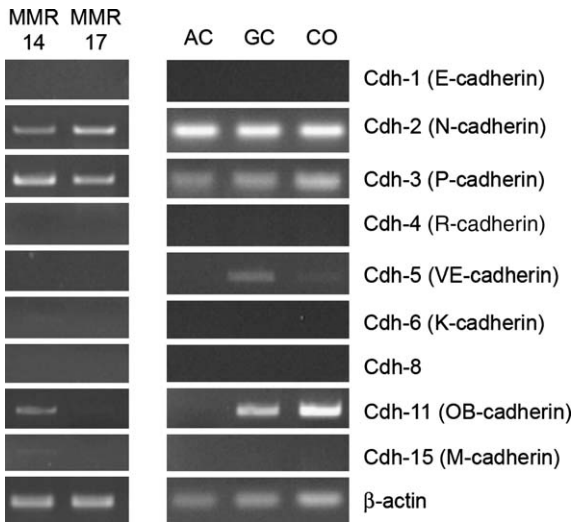

Fig. 4. Expression of cadherin family genes. The expression of cadherin family genes in cell lines and primary cultured articular chondrocytes, growth plate chondrocytes and COs was examined by RT-PCR.

SUPPRESSION OF CDH-11 GENE EXPRESSION INHIBITED THE FORMATION OF NODULES BY MMR14 CELLS

To further investigate the role of Cdh-11 in the formation of nodules by MMR14 cells, the expression of the gene was suppressed by siRNA. Three weeks after the transduction of the lentiviral vector expressing siRNA for the Cdh-11 gene (LV-Cdh11i) or firefly luciferase gene (LV-FFi), the expression of the Cdh-11 gene was analyzed by RT-PCR. LV-Cdh11i-transduced MMR14 cells (MMR14/LV-Cdh11i) expressed much less of the Cdh-11 gene than parental MMR14 cells or LV-FFi-transduced MMR14 cells (MMR14/LV-FFi) [Fig. 6(A)]. The expression level of Alp

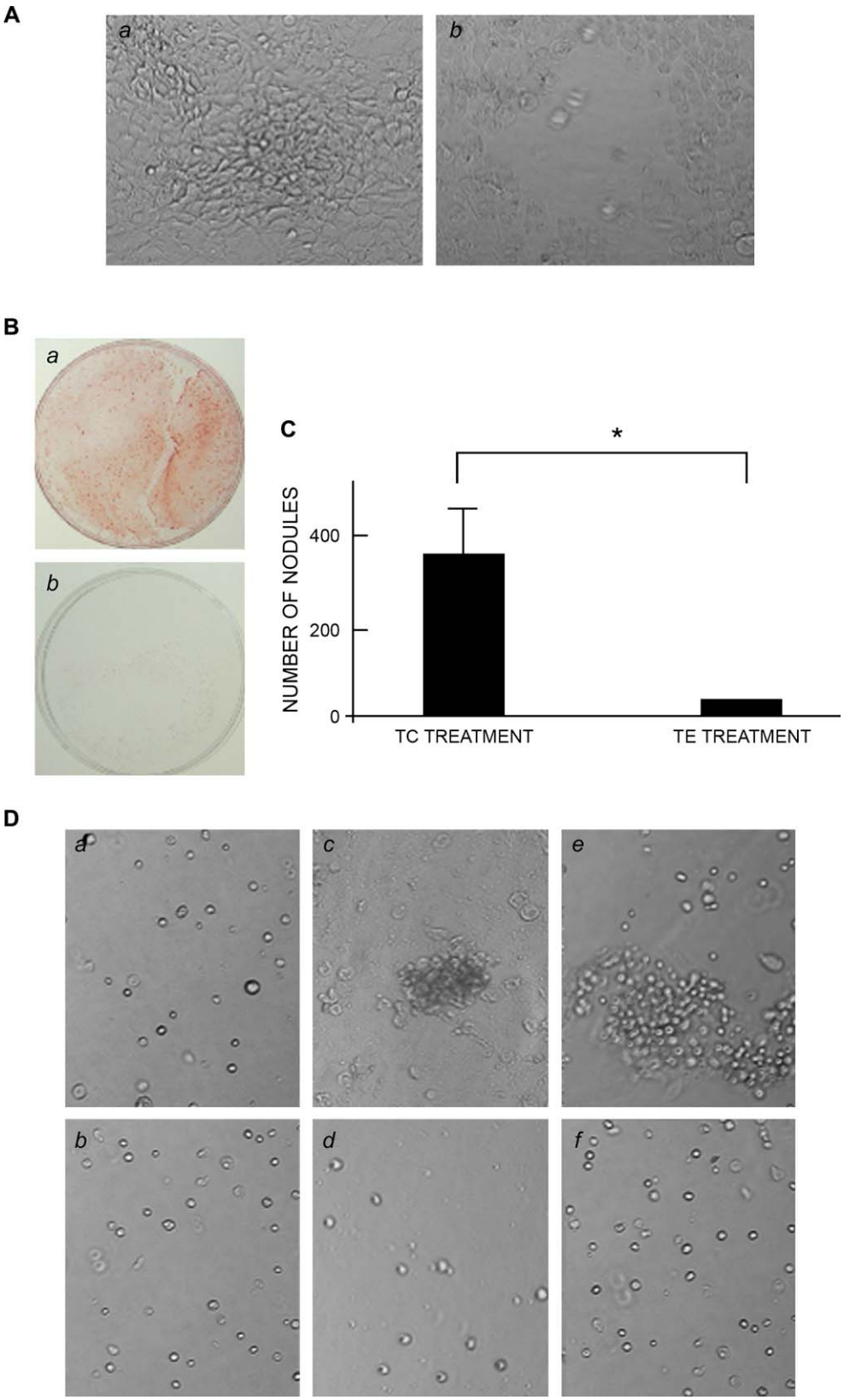

Fig. 5. Cell−cell adhesion of MMR14 requires Ca^{2+}. MMR14 cells were cultured for 2 weeks to form nodules, and then treated with 0.01% trypsin with (TC treatment) (A, *a* and B, *a*) or without (TE treatment) (A, *b* and B, *b*) 1 mM $CaCl_2$. (A, *a* and *b*) Phase contrast view of nodules. (B, *a* and *b*) Alizarin red staining of nodules. (C) The number of alizarin red-positive nodules after TC or TE treatment. Experiments were performed individually two times. $^*P < 0.01$. MMR17 cells (D, *a* and *b*), MMR14 cells (D, *c* and *d*), and primary cultured chondrocytes from growth plate (D, *e* and *f*), were dispersed with 0.01% trypsin without $CaCl_2$. After a wash with HCMF, cells were allowed to aggregate in HCMF with (D, *a*, *c*, and *e*), or without (D, *b*, *d*, and *f*) 1 mM $CaCl_2$ (original magnification, $\times$100).

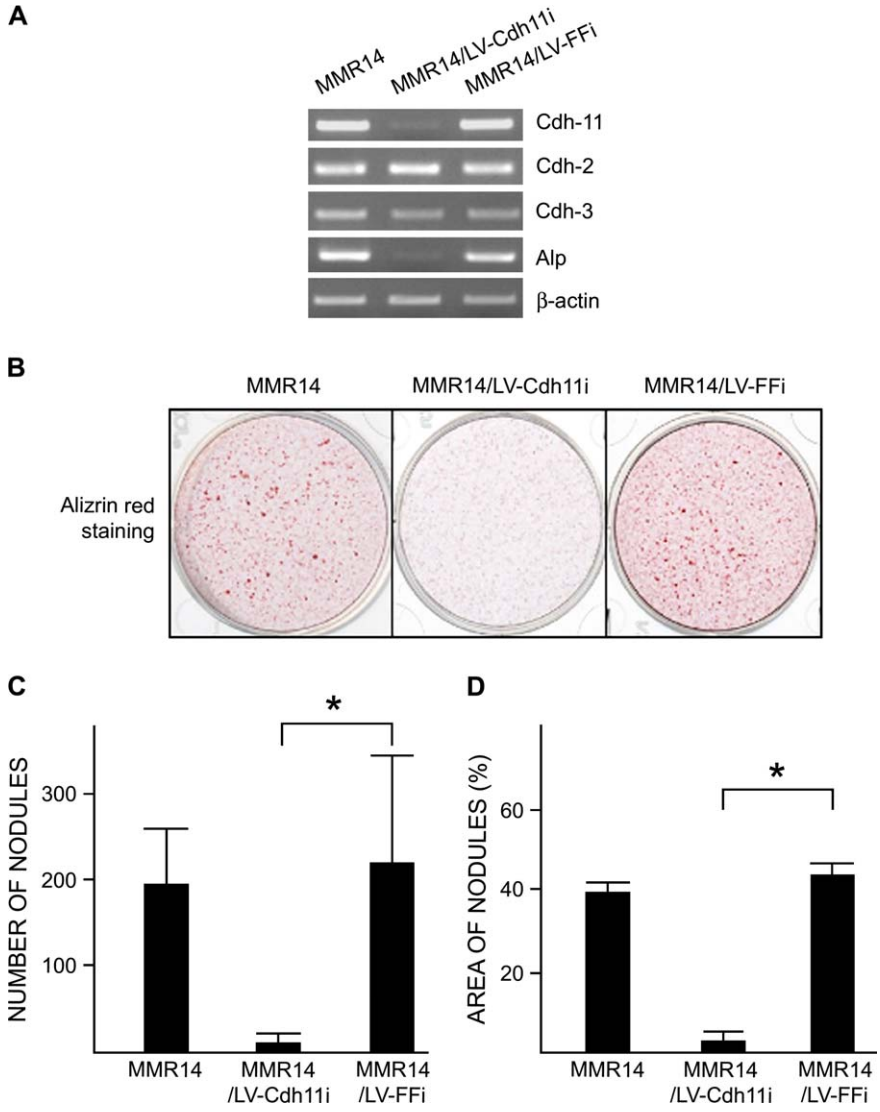

Fig. 6. Knockdown of the expression of Cdh-11 by siRNA reduced the nodular formation of MMR14. The mRNA expression of the Cdh-11, Cdh-2, Cdh-3 and Alp genes was analyzed by RT-PCR in MMR14, MMR14 transduced with lentiviral expression vectors containing siRNA for the firefly luciferase gene (MMR14/LV-FFi), or the murine cadherin-11 gene (MMR14/LV-Cdh11i) (A). Calcified nodules produced by each cell were stained with alizarin red (B) and the number (C) and percent area (D) of alizarin red-positive nodules were calculated. $^{*}P < 0.01$, $^{*}P < 0.01$.

was also significantly reduced in MMR14/LV-Cdh11i [Fig. 6(A)]. The expression level of the Cdh-2 and Cdh-3 genes showed no difference between MMR14, MMR14/LV-Cdh11i, and MMR14/LV-FFi, indicating specific suppression of the Cdh-11 gene by LV-Cdh11i [Fig. 6(A)]. MMR14/LV-Cdh11i produced a fewer nodules than MMR14 or MMR14/LV-FFi [Fig. 6(B)], and the statistical analyses indicated that the number and percent area of calcified nodules in MMR14/LV-Cdh11i were significantly reduced [Fig. 6(C and D)]. To confirm the association of Cdh-11 expression with the ability to form nodules, several stable clones were isolated, and the downregulation of the Cdh-11 gene in each clone was analyzed and quantified [Fig. 7(A)]. The number of calcified nodules produced by each clone was well correlated with the expression level of the Cdh-11 gene in each clone [Fig. 7(A and B)]. These data strongly suggested that the expression of the Cdh-11 gene is required for nodules to form in MMR14.

INDUCTION OF Cdh-11 FAILED TO INDUCE THE FORMATION OF NODULES BY MMR17 CELLS

To investigate whether Cdh-11 is capable of inducing the formation of nodules in MMR17, the expression of its gene was induced using the retroviral expression vector containing the full-length cDNA of the murine Cdh-11 gene (pQCXIH-Cdh11). After 3 weeks of culture, the expression of Cdh-11 RNA [Fig. 8(A)] and protein [Fig. 8(B)] was detected in MMR17 cells transduced with pQCXIH-Cdh11 (MMR17/pQCXIH-Cdh11). The expression level of the Alp gene in MMR17/pQCXIH-Cdh11 was slightly increased, but other bone-related genes showed no significant change [Fig. 8(A)]. Expression of the Cdh-11 protein at cell–cell junctions in MMR17/pQCXIH-Cdh11 was confirmed by immunocytochemistry [Fig. 8(C)]. The formation of nodules, however, was not observed in MMR17/pQCXIH-Cdh11 or in the control (MMR17/pQCXIH) [Fig. 8(D)]. These results

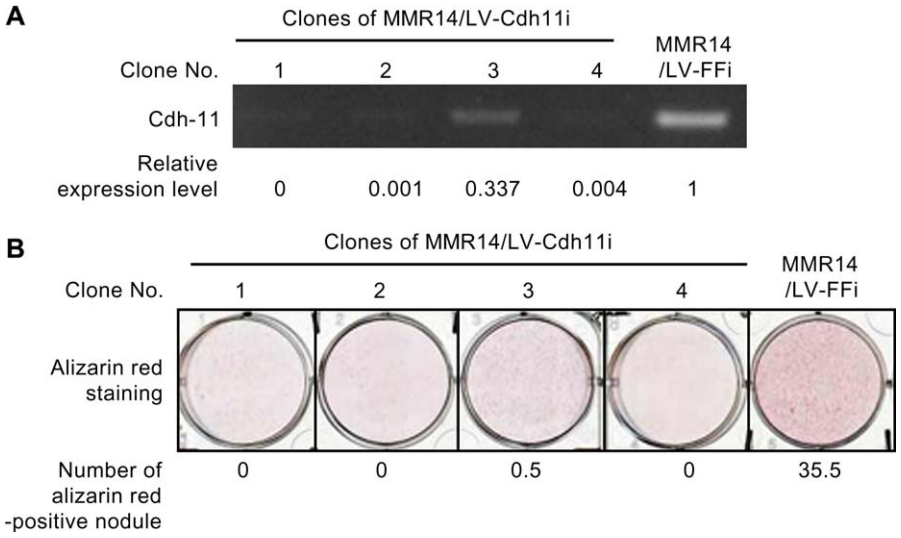

Fig. 7. Association of expression level of the Cdh-11 gene with the number of calcified nodules in siRNA-transduced clones. MMR14 cells transduced with LV-Cdh11i were cultured under drug selection, and single cell cloning by the limited dilution method was performed. Four clones were randomly selected and further analyzed. The mRNA expression of the Cdh-11 gene of each clone was analyzed by RT-PCR (A, upper), and the relative expression level was digitalized as described in the Materials and methods section (A, lower). Each clone was cultured for 2 weeks to produce nodules, which were then stained with alizarin red (B, upper). The number and percent area of alizarin red-positive nodules were calculated (B, lower).

suggested that Cdh-11 is indispensable, but not the sole factor, to induce nodular formation.

Discussion

Gene expression profiling of MMR14 and MMR17 provided a list of interesting genes. Among the genes upregulated in MMR14 cells, some have been known to promote calcification in the growth plate. Mmp13 is a member of the Mmp family of neutral endopeptidases, and Mmp13 null mice showed profound defects in growth plate cartilage[23,24]. Alp is expressed from the hypertrophic zone to calcifying zone in growth plate cartilage[25], and osteoblasts in Alp null mice differentiate normally but are unable to initiate mineralization *in vitro*[26]. Several extracellular matrix (ECM) genes were also identified as upregulated in MMR14. Col1a1 is a well known marker for osteogenesis, and Col11a1 is so essential for the normal formation of cartilage collagen fibrils that mice, which are homozygous for the autosomal recessive chondrodysplasia mutation, die at birth with abnormalities in the cartilage of limbs, ribs, mandible, and trachea[27]. Fibronectin is also expressed in the hypertrophic zone[25]. Fibulin-1 is a Ca^{2+}-binding ECM protein associated with fibronectin, aggrecan and versican[28,29], and expressed in the hypertrophic zone of growth plates[29], although its precise function in the growth plate is unknown. On the other hand, some genes upregulated in MMR17 cells are known to prevent calcification. Virally driven overexpression of the Mgp gene in cultured chondrocytes decreased mineralization[30]. Mice deficient in Mgp showed calcification of the arteries[31], and mice deficient in both Mgp and Opn had twice as much arterial calcification as Mgp (−/−) Opn (+/+) mice, suggesting that Mgp and Opn inhibit the calcification *in vitro* and *in vivo*[31]. The relation between Enpp2 and calcification is unclear, but a closely-related gene, Enpp1, regulates ectopic ossification[32–34]. CD151 is a member of the tetraspanin superfamily, and plays a key role in the regulation of integrin-mediated cell migration[35–37]. The identification of these genes, the proposed functions of which agreed with the phenotype of each cell line, endorsed the reliability of our gene expression profiling, and we focused on one gene, Cdh-11, because of an absence of data on its expression in cartilage tissue.

Cadherins are integral membrane glycoproteins that provide strong intercellular adhesion via homophilic molecular associations in a Ca^{2+}-dependent manner[38,39]. Cadherin genes form a gene superfamily that consists of two large subfamilies, classical and atypical cadherins, the former being further divided into type 1 and type 2[39]. Cdh-11 is a type 2 classic cadherin isolated as an osteoblast-specific gene by subtraction screening between osteoblasts and liver cells[18,39]. Subsequent analyses revealed that Cdh-11 regulates osteoblastic differentiation in cell–cell adhesion and formation of calcified nodules[19,20]. Recently, it was proposed that in addition to its function in the formation of matrices, Cdh-11 may promote the process by which undifferentiated mesenchymal cells turn into osteogenic-lineage cells[20,40]. When C2C12 cells were forced to express Cdh-11, they began to express osteogenic markers such as Alp and Fgfr2[40]. We observed that the expression of the Alp gene was downregulated by siRNA for the Cdh-11 gene [Fig. 6(A)] and upregulated by forced expression of the Cdh-11 gene [Fig. 8(A)], which may be consistent with previous findings.

As far as we know, no study has precisely analyzed the significance of Cdh-11 in cartilage tissues and chondrocytes. The results of the cell aggregation assay and siRNA experiments clearly indicated that the function of Cdh-11 is indispensable for the formation of calcified nodules. Cdh-11 knockout mice, however, showed only a slight reduction in bone density and an almost normal development of the limb[19]. Cdh-2 (N-cadherin) knockout mice also had normally developed limbs[41]. Functional redundancy of Cdh-11 and Cdh-2 was proposed to explain the discrepancy between *in vivo* and *in vitro* experiments[19,41]. In the present

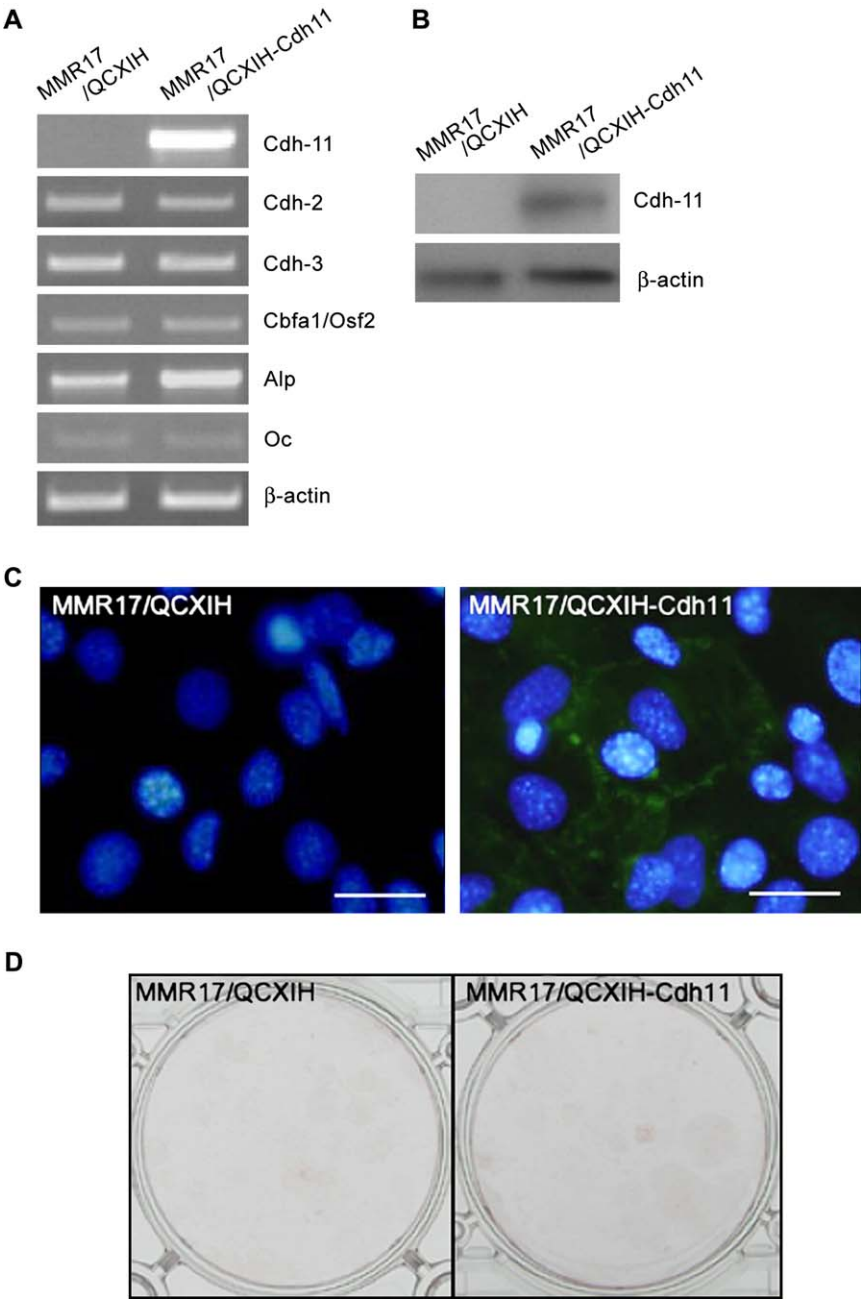

Fig. 8. Introduction of Cdh-11 expression in MMR17. The mRNA expression of cadherin and calcification-related genes was analyzed by RT-PCR in MMR17 transduced with a retroviral expression vector containing the murine Cdh-11 gene (MMR17/QCXIH-Cdh11) or an empty vector (MMR17/QCXIH) (A). The expression of Cdh-11 protein in these cells was analyzed by Western blotting (B) and immunocytochemistry (C) (original magnification, $\times$400; bar = 10 μM). Calcified nodule formation of these cells was analyzed by alizarin red staining (D).

study, however, the formation of nodules in MMR14 was severely inhibited by the downregulation of Cdh-11 by siRNA, which induced no change in the Cdh-2 gene expression. Forced expression of Cdh-11 in MMR17 failed to induce nodular formation. Therefore, it is clear that molecule(s) other than Cdh-11 and Cdh-2 are also involved in the processes of nodular formation and calcification.

It is paradoxical that cadherin is a cell–cell adhesion molecule, whereas chondrocytes in hypertrophic zone do not form cell–cell attachment. In contrast with the strong expression of Cdh-11 in primary cultured GCs (Fig. 2), only some cells in calcified chondrocytes were positive for the Cdh-11 (Fig. 3), suggesting that the release from lacunae may induce the expression of the Cdh-11 gene in cells in the growth plate. Such an induction was not a nonspecific phenomenon *in vitro*, because it was not observed in articular chondrocytes (Fig. 2). Chondrocytes are classified as either permanent or transient based on their potential for calcification, and it is important to identify which molecules promote calcification in transient chondrocytes (growth plate cartilage) or prevent the calcification of permanent chondrocytes (articular cartilage)[42,43]. As shown in this study, chondrocytes

isolated from growth plate expressed the Cdh-11 gene, whereas primary cultured chondrocytes from AC as well as MMA2 cells, an immortalized articular chondrocyte cell line[15], showed no expression of the Cdh-11 gene even after prolonged *in vitro* culture (data not shown). MMA2, but not MMR14, expressed the C-1-1 gene[15], which is a marker of permanent chondrocytes[43,44]. These results suggested that the ability to express the Cdh-11 gene is one of the features of transient chondrocytes, and therefore molecules controlling the expression of the Cdh-11 gene in chondrocytes may determine their fate. In relation to this issue, the negative expression of the Cdh-11 gene in MMR17 is intriguing. We have no clear explanation for the difference in the expression level of the Cdh-11 gene between MMR14 and MMR17. Cells without a p53 gene are known to have genetic instability, and MMR17 may have a defect in the Cdh-11 gene, although no gross rearrangement was found (data not shown). Treatment with 5-azacytidine failed to induce the expression of the Cdh-11 gene, excluding the possibility of methylation-induced gene silencing (data not shown). Immunohistochemical analyses in growth plates showed that only cells in the late hypertrophic and calcified zones exhibited positive staining of the Cdh-11 gene, and most cells in the hypertrophic zone were negative. These results suggested that although the two cell lines shared a similar gene expression profile as cells in the hypertrophic zone[16], they may represent cells in different stages of differentiation in the hypertrophic zone, and that factor(s) promoting the differentiation may decide the different properties of MMR14 and MMR17.

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